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(54) Title: PREVENTION OR TREATMENT OF DEFICITS THAT ARISE IN CONNECTION WITH DISEASES OF, OR INJURIES TO, THE NERVOUS SYSTEM

(57) Abstract: The present invention includes compositions and methods for use in, for example, maintaining or increasing synaptic plasticity, strengthening synaptic transmission, and/or treating or preventing motor, sensory, or cognitive deficits (including enhancing memory and learning). The compositions and methods of the invention can be used to treat, for example, injuries to the nervous system (such as spinal cord injuries), a variety of neural and neurodegenerative diseases, including those associated with dementia (e.g., Alzheimer's disease), and disorders that interfere with learning or impair memory.

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Prevention or Treatment of Deficits That Arise in Connection With Diseases of, or Injuries to, the Nervous System

PRIORITY CLAIM

This application claims the benefit of the priority date of U.S.S.N. 60/480,112, which was filed June 20, 2003. For the purpose of any patent(s) that may issue from the present application in the United States of America, the entire content of the prior application is hereby incorporated by reference in the present application.

RELATED PATENTS AND PATENT APPLICATIONS

The disclosure of the present patent application is related to the disclosures of United States Patent Application Serial Nos. 09/997,848 and 10/296,551, and of United States Provisional Patent Application Serial Nos. 60/250,079, 60/385,230, 60/442,030, and 60/480,112, especially as their disclosures relate to making and using HSV amplicons. For the purpose of any patent(s) that may issue from the present application in the United States of America, these patent applications, and any patent applications from which they claim priority, or which presently claim priority to them, are hereby incorporated by reference in the present patent application in their entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

Some of the work described here was funded by a grant from the National Institutes of Health (R01AG020204). The United States government may, therefore, have certain rights in the invention.

TECHNICAL FIELD

The present invention relates to compositions and methods for treating patients who have a disease, or who have suffered a traumatic injury, that affects the nervous system. More particularly, the invention relates to compositions, including amplicon particles, that mediate the expression of all or part of an NMDA receptor and to the administration of neurotrophins to improve (by, for example, preventing, inhibiting, or attenuating) sensory, motor, or cognitive deficits. Methods of making and using amplicon particles to achieve such improvement are also within the scope of the present invention.

BACKGROUND

Motoneurons and their synaptic inputs develop considerably during the first two postnatal weeks as adult motor patterns are established (Walton *et al.*, *Neuroscience* 51: 763, 1992; Jamon and Clarac, *Behav. Neurosci.* 112:1218, 1998; Lanuza *et al.*, *J. Neurosci. Res.* 67:607, 2002). Plasticity of synaptic inputs to spinal motoneurons during this perinatal period plays an important role because activity affects motor neuron development (Hockfield and Kalb, *Curr. Opin. Neurobiol.* 3:87, 1993; Kalb and Hockfield, *Dev. Biol.* 162:539, 1994), maturation of motor behavior (Vinay *et al.*, *Brain Res. Bull.* 53:635, 2000), and regulation of motor neuron dendrite growth (Kalb, *Development* 120:3063, 1994).

Motoneurons in neonatal rat spinal cord can be activated monosynaptically by two input fiber systems: a segmental input via dorsal root (DR) afferents, and a descending input via fibers in the ventrolateral funiculus (VLF) (Fulton and Walton, *J. Physiol. (Lond.)* 370:651, 1986; Pinco and Lev-Tov, *J. Neurophysiol.* 72:2406, 1994). Monosynaptic transmission from both inputs occurs via glutamate activation of AMPA/kainate receptors (Pinco and Lev-Tov, *J. Neurophysiol.* 72:2406, 1994; Pinco and Lev-Tov, *J. Neurophysiol.* 70:406, 1993).

SUMMARY OF THE INVENTION

The present invention is based, in part, on studies we conducted to examine the loss of plasticity in neurons. The studies involve neurotrophins, which are growth factors that play important roles in developing animals, and the N-methyl-D-aspartate (NMDA) receptor. We found, *inter alia*, that the neurotrophin NT-3 strengthens synaptic responses of lumbar motoneurons at descending inputs in the rat fetus, but not in postnatal rats. The loss of NT-3 action over this developmental period is influenced by Mg^{2+} blockade of the NMDA receptor, which must function in order for NT-3 to exert its action on a cell. The Mg^{2+} blockade of NMDA receptors occurs concurrently with a decrease in the expression of a subunit of the NMDA receptor, NR2D.

We also found that delivery of NR2D to motoneurons in animals delays the age-related development of Mg^{2+} blockade and extends the action of NT-3 to postnatal animals. This reveals a previously unknown molecular link between NR2D subunit expression and Mg^{2+} blockade, and it provides evidence that neurotrophins such as NT-

3 can strengthen synaptic transmission in postnatal motoneurons when NR2D expression is maintained.

Accordingly, the invention features compositions that can be used to deliver a neurotrophin (*e.g.*, NT-3 or a biologically active variant thereof) and/or an NMDA receptor, a subunit thereof (*e.g.*, an NR2D subunit), or biologically active variants thereof, to cells within the nervous system (*e.g.*, neurons in the peripheral or central nervous system). These compositions can include a vector such as a DNA vector (*e.g.*, a plasmid, cosmid, BAC, YAC, or the like); a viral vector such as a retroviral vector or a vaccinia viral vector (*e.g.*, a modified vaccinia Ankara vector); or an amplicon particle, such as those described further below, that expresses the neurotrophin and/or the NMDA receptor or subunit (*e.g.*, NR2D). In alternative embodiments, other NMDA receptor subunits (or biologically active variants thereof) or entire NMDA receptors (or biologically active variants thereof) can be delivered and expressed. For ease of reading, we do not repeat the phrase "or biologically active variants thereof" every time we refer to an NMDA receptor or a part thereof (*e.g.*, a whole or partial subunit, including a mutant subunit). In any circumstance where an NMDA receptor or a part or portion thereof is useful, a biologically active variant can be used either in addition to, or instead of, its naturally occurring counterpart.

The neurons targeted by the compositions of the invention can be motoneurons or other neurons that naturally express NMDA receptors (although the invention is not so limited; neurons other than motoneurons and those that express NMDA receptors can be targeted as well).

As noted above, all or part of an NMDA receptor can be delivered to a cell (*e.g.*, a neuron in the central nervous system (CNS), such as a motoneuron) by way of an expression vector, many of which are known in the art. NR2D subunits, for example, can be delivered by an amplicon particle, such as those made by the helper virus-free methods described below. One of ordinary skill in the art can refer to, and use, published methods of making amplicon expression vectors as well. For example, the methods of the present invention can be carried out using herpes virus amplicon particles as described in, for example, WO 01/89304 (entitled "Method of Producing Herpes Simplex Virus Amplicons, Resulting Amplicons, and Their Use") and in WO 02/056828 and WO 03/101396 (both entitled "Helper Virus-Free Herpesvirus Amplicon Particles and Uses Thereof"). The transgene included, however, would be a

transgene as described herein and suitable for use with the present methods (*e.g.*, a sequence encoding NR2D or a biologically active variant thereof; *see, e.g.*, Hess *et al.*, *J. Neurochem.* 70:1269-1279, 1998; suitable sequences are also available through GenBank (*see, e.g.*, GenBank Accession No. O15399).

Thus, the invention includes: methods of making helper virus-free amplicon particles (*e.g.*, herpes simplex virus (HSV) amplicon particles) that include a sequence encoding an NMDA receptor or a subunit thereof and/or a neurotrophin; isolated cells that contain those particles (*e.g.*, packaging cell lines or primary cells (*e.g.*, a patient's cells), infected *in vivo* or *ex vivo*); particles produced according to those methods (such particles, regardless of the method by which they are produced, may be abbreviated herein as "hf-HSV" particles so long as the method is carried out without using helper virus); and methods of treating a patient with an hf-HSV particle made according to those methods. For example, hf-HSV particles (including those made according to the methods described herein) that contain one or more genes encoding one or more therapeutic proteins (*e.g.*, one or more subunits of an NMDA receptor or one or more neurotrophins (*e.g.*, NT3)) can be used to transduce cells in the CNS (*e.g.*, in neuron in the brain or spinal cord).

The hf-HSV particles, regardless of the precise polypeptide they encode, can be made according to methods known in the art. For example, the hf-HSV particles can be made according to the methods described below (*see also* United States Patent Application Serial Nos. 09/997,848 and 10/296,551, and United States Provisional Patent Application Serial Nos. 60/250,079, 60/385,230, 60/442,030).

The compositions of the present invention can be used to treat patients whose nervous systems have been compromised by injury or disease. For example, the methods of the invention can be used to a treat a patient identified as being paralyzed (to any extent) as the result of trauma or who has a disease that produces sensory deficits (such as those that occur in the event of cerebral palsy), motor deficits (such as those that occur in the event of Amyotrophic Lateral Sclerosis), or cognitive deficits, including dementia (as occurs in the event of Alzheimer's Disease). Of course, many diseases produce more than one type of deficit (*e.g.*, patients with Huntington's Disease may experience motor and cognitive deficits). The deficit can also be one associated with a congenital or birth defect (*e.g.*, hydrocephalus or spina bifida) or caused by

trauma to a fetus or infant, including surgical trauma. Other patients amenable to treatment are described further below.

As noted above, the invention features methods of generating a herpesvirus amplicon particle. In one embodiment, the method includes providing a cell that has been stably transfected with a nucleic acid sequence that encodes an accessory protein (alternatively, a transiently transfected cell can be provided). That cell is transfected with (a) one or more packaging vectors that, individually or collectively, encode one or more herpesvirus (*e.g.*, HSV) structural proteins but do not encode a functional herpesvirus cleavage/packaging site and (b) an amplicon plasmid that includes a sequence that encodes a functional herpesvirus cleavage/packaging site, a herpesvirus origin of DNA replication, and a sequence encoding a therapeutic agent of the invention (*e.g.*, an NR2D subunit of an NMDA receptor, NT-3, or biologically active variants thereof). We do not repeat all of the possibilities for the therapeutic agent in every instance. For example, the sequence included in the vector can be that of a subunit of a human NMDA receptor, and it is to be understood that such sequences are suitable for incorporation in all configurations of the present invention (*e.g.*, a human NR2D or NT-3 sequence can be used with any amplicon (whether or not of HSV-1 and whether or not made by a "helper virus-free" method) or conventional plasmid expression vector). Similarly, the therapeutic agent can be, in any configuration of the compositions of the present method, a biologically active variant of an NMDA receptor, a subunit thereof, or a neurotrophin (*e.g.*, NT-3). Biologically active variants differ from their wild-type counterparts (in, for example, sequence or post-translational modification) but retain the ability to function in the methods of the invention (*i.e.*, they retain the ability to serve as therapeutic agents by improving a sign or symptom of a neural deficit (*e.g.*, a motor, sensory, or cognitive deficit) to any clinically beneficial extent. The variant may retain all of the perceived biological activity of the wild-type counterpart and may, indeed, may be more active. With respect to polypeptide therapeutics, any of the variants described herein can differ from their wild-type counterparts by virtue of containing one or more additional amino acid residues, which may be added to either terminus of the polypeptide and/or interspersed in the sequence), one or more deletions, which may be made from either or both termini or from within the sequence, or one or more amino acid substitutions (*e.g.*, conservative or non-conservative amino acid substitutions). A combination of such changes may be

present in any given variant. While only one residue may be changed (or only a few residues (*e.g.*, 2, 3, 4, 5, or 6), more substantial variants may also have biological activity (*e.g.*, variants that vary from their wild-type counterparts by addition, deletion, or substitution of 1-10% (*e.g.*, 2, 4, 5, 7, or 10) of the amino acid residues or more (*e.g.*, 12, 15, 20, 25, 30, 40, or 50% of the residues may be deleted or substituted).

In another embodiment, the method is carried out without a stably transfected cell. For example, a cell is transfected with (a) one or more packaging vectors that, individually or collectively, encode one or more HSV structural proteins but do not encode a functional herpesvirus cleavage/packaging site; (b) an amplicon plasmid comprising a sequence that encodes a functional herpesvirus cleavage/packaging site, a herpesvirus origin of DNA replication, and a sequence that encodes a therapeutic agent of the present invention; and (c) a nucleic acid sequence that encodes an accessory protein.

The variants described elsewhere herein (*e.g.*, the various suitable therapeutic agents, expression vectors, and patients amenable to treatment) can be incorporated in methods carried out with stably or transiently transfected cells. For example, in either of these methods, or any other known in the art, one or more of the following additional limitations may apply. The herpesvirus can be any of the more than 100 known species of herpesvirus. For example, the herpesvirus can be an alpha herpesvirus (*e.g.*, a Varicella-Zoster virus, a pseudorabies virus, or a herpes simplex virus (*e.g.*, type 1 or type 2 HSV) or an Epstein-Barr virus. Similarly, the accessory protein can vary and can be a protein that inhibits the expression of a gene in the cell. For example, the accessory protein can be a virion host shutoff (vhs) protein (*e.g.*, an HSV-1, -2, or -3 vhs protein, bovine herpesvirus 1 vhs protein, bovine herpesvirus 1.1 vhs protein, gallid herpesvirus 1 vhs protein, gallid herpesvirus 2 virion hsp, suid herpesvirus 1 vhs protein, baboon herpesvirus 2 vhs protein, pseudorabies vhs protein, cercopithecine herpesvirus 7 vhs protein, meleagrid herpesvirus 1 vhs protein, equine herpesvirus 1 vhs protein, or equine herpesvirus 4 vhs protein). Any of these proteins can be operatively coupled to its native transcriptional control element(s) or to an artificial control element (*i.e.*, a control element that does not normally regulate its expression *in vivo*).

The methods by which herpesvirus amplicon particles are generated can also include a step in which the cell is transfected with a sequence encoding a VP16 protein,

which may be transiently or stably expressed. Alternatively, or in addition, one can engineer a transcriptional activator to mimic VP16 (*e.g.*, a pseudo-activator that recognizes *cis* elements but uses a different transcriptional activation domain).

In any configuration of the compositions and methods of the invention, the VP16 protein can be, for example, HSV-1 VP16, HSV-2 VP16, bovine herpesvirus 1 VP16, bovine herpesvirus 1.1 VP16, gallid herpesvirus 1 VP16, gallid herpesvirus 2 VP16, meleagrid herpesvirus 1 VP16, or equine herpesvirus 4 VP16.

The vhs and VP16 encoding sequences can be introduced into a cell on the same vector or on two different vectors or on two different types of vectors (*e.g.*, both sequences can be introduced in the same plasmid, in two different plasmids, or in a plasmid and cosmid). Sequences encoding vhs and/or VP16 can be transiently or stably introduced into cells (these methods are routine in the art), and the invention features isolated cells that are transiently or stably transfected with one or both of the sequences that encode one or more of a vhs or VP16 protein. These cells may be, or may subsequently be, transfected with any of the other components described above (*e.g.*, packaging vectors and/or amplicon plasmids).

As noted above, the herpesvirus (*e.g.*, HSV) amplicon particles can be made by methods that employ one or more packaging vectors, which may comprise a cosmid (and may include a set of cosmids), a yeast artificial chromosome, a bacterial artificial chromosome, a human artificial chromosome, or an F element plasmid. A single packaging vector can encode large portions of the genome of a herpesvirus (and up to the entire genome), or the genome may be divided between two or more vectors. For example, the packaging vectors can include a set of cosmids (*e.g.*, a set of cosmids comprising cos6Δa, cos28, cos14, cos56, and cos48Δa). One or more packaging vectors, individually or collectively, can express the structural herpesvirus proteins. The herpesvirus origin of DNA replication is not present in the one or more packaging vectors.

In the context of the present invention, the therapeutic agent encoded by the amplicon plasmid is expected to be a protein that constitutes at least a part of an NMDA receptor (*e.g.*, a subunit of the receptor) or a neurotrophin (we use the term "protein" as it is conventionally used, to denote a polymer of amino acid residues, which may be naturally or non-naturally occurring and which may be modified (*e.g.*, glycosylated)). The protein can be expressed by an amplicon plasmid that includes a promoter that

increases the expression of the therapeutic agent. The promoter can be heterologous to the therapeutic gene (*e.g.*, any known constitutively active promoter can be used to drive expression of NR2D) or it can be the promoter that drives the expression of the therapeutic gene in a natural genome (*e.g.*, the promoter that drives expression of NR2D can be the NR2D promoter; the promoter that drives NT-3 expression can be a naturally occurring NT-3 promoter). The amplicon plasmid can also contain other regulatory elements, such as enhancers or binding sites for various transcription factors (*e.g.*, a cyclic AMP-response element ((CRE).

One or more of the compositions of the invention can be packaged as a kit provided to study neuronal plasticity or to treat a patient who has a deficit associated with impaired neural function (whether as a consequence of a disease process or an injury; we use the term "patient" to denote any animal subjected to the methods of the invention, whether a laboratory or domesticated animal or a human patient, including a human fetus, newborn, child, or adult). The kits can contain one or more of the herpesvirus amplicon particles or other types of vectors described herein; one or more of the cells containing them; or one or more of the components useful in generating either the particles or the cells. For example, a kit can include a packaging vector and an amplicon plasmid. Optionally, the kit can also contain stably transfected cells. Optionally, the kit can include instructions for use (*e.g.*, printed instructions). Where the kit is provided to treat patients, the amplicon particles can be formulated as pharmaceutically acceptable compositions (*e.g.*, contained within a buffered solution or another physiologically acceptable carrier or diluent; the pharmaceutical compositions of the invention are described further below).

Therapeutic vectors based on the herpes simplex virus have a number of features that lead us to believe they will be advantageous. For example, they exhibit a broad cellular tropism, they have the capacity to package large amounts of genetic material (and thus can be used to express multiple genes or gene sequences), they have a high transduction efficiency, and they are maintained episomally, which makes them less prone to insertional mutagenesis. Moreover, HSV vectors can transduce non-replicating or slowly replicating cells, which has therapeutic advantages and expands the research uses of the present compositions. For example, freshly isolated cells can be transduced in tissue culture, where conditions may not be conducive to cell replication. The ability of HSV vectors to infect non-replicating or poorly replicating

cells also means that they are especially well suited for use in the nervous system; most neurons fail to divide once they are phenotypically mature. The transduction procedure can also be carried out fairly quickly.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, useful methods and materials are described below. All publications (including those with both full and partial citations of page number ranges), patent applications (and patent applications or patents from which they claim priority or which claim priority from them), patents (and patent applications or patents from which they claim priority or which claim priority from them), and other references mentioned herein are incorporated by reference in their entirety. In case of conflicting subject matter, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a series of graphical representations showing age-related changes of the effect of NT-3 on monosynaptic transmission and the properties of NMDA-mediated responses during perinatal period.

FIG. 1A is a graph showing Mg^{2+} sensitivity of NMDA responses. DR and VLF responses in the same MN from 12 day-old, 2 day-old, and E18 animals, respectively. Numeric labels for 12 day-old DR: (1) AMPA/kainate monosynaptic responses in normal ACSF (2 mM Mg^{2+}, dashed); (2) 15 minutes after administration of non-NMDAR antagonists (AMPA/kainate antagonist CNQX- 10 mM, GABAA antagonist bicuculline - 5 mM, GABAB antagonist CGP35348- 5 mM, glycine antagonist strychnine- 5 mM), 2mM Mg^{2+}; (3, 4) NMDA responses with monosynaptic component (at arrow) after decrease of Mg^{2+} concentration in saline to 500 μM and 100 μM, respectively. Each of the remaining 5 groups of 4 records in are not labeled but are organized in the same way: record 1 is dashed; peak (record 2) < peak (record 3) < peak (record 4).

FIG. 1B is a graph showing the percentage change in the peak amplitude of monosynaptic NMDAR-mediated responses evoked by stimulation of DR or VLF in 1-

5 day-old, 8-15 day-old, and E18 rats as a function of extracellular Mg^{2+}. Data points are mean ± SE of responses recorded from 6-8 cells for each case and normalized to maximum (100%) response obtained in 50 μM Mg^{2+} solution. Regression curves were drawn by 3-parameter Hill equation. KMg is the dissociation constant for Mg^{2+} determined by this regression analysis.

FIG. 1C is a graph showing the percentage change in peak amplitude of monosynaptic AMPA/k - responses evoked by stimulation of DR or VLF in 1-5 day-old rats as a function of extracellular Mg^{2+} (n = 5).

FIG. 1D is a graph showing superimposed DR and VLF responses of same MN from E18 rat, in normal ACSF and 15 minutes after bath administration of 0.2 μg/ml NT-3.

FIG. 1E is a graph showing data to demonstrate the age-related changes in the effect of bath-applied 0.2 μg/ml NT-3 on peak amplitude of monosynaptic AMPA/kainate EPSP at DR and VLF synapses. Note that NT-3 facilitates both DR- and VLF-evoked responses at E18 (n = 4). Data for 1 week and 2 week is a summary of results presented earlier (Arvanov *et al.*, *J. Neurophysiol.* 84:752, 2000).

FIG. 2 is a series of graphs describing how delivery of NR2D via an HSV amplicon vector enhanced expression of NR2D subunits, decreased Mg^{2+} blockade of NMDA receptor and re-instated the ability of NT-3 to facilitate both DR and VLF responses in 2 week-old animals.

FIG. 2A is a graph showing the percentage change in the peak amplitude of monosynaptic NMDAR-mediated responses evoked by stimulation of DR or VLF in 10-12 day old rats injected with HSVnr2d or a β-galactosidase expressing control amplicon (HSVlac) at postnatal day 2, as a function of extracellular Mg^{2+} (see FIGS. 1A-E for details). Horizontal arrows are inserted to facilitate comparison of control and NR2D virus treatment.

FIG. 2B is a graph showing the results of real time quantitative RT-PCR analyses to demonstrate the enhanced level of expression of NR2D subunits after virus treatment.

FIG. 2C is a graph showing superimposed DR and VLF responses in normal ACSF and 15 minutes after bath administration of 0.2 μg/ml NT-3 in 12 day-old rats injected with HSVnr2d or HSVlac, respectively.

FIG. 2D is a graph showing data demonstrating that 0.2 μg/ml NT-3 facilitates both DR- and VLF-evoked fast monosynaptic in 10-12 day-old rats injected at day 2 with HSVnr2d (n = 7), but not HSVlac control (n = 6). Asterisks indicate significance at the 0.05 level after Bonferroni's correction used to adjust for multiple comparisons.

FIG. 3 is a table identifying the essential HSV-1 genes.

FIG. 4 is a table showing a comparison of NMDA receptor subunit mRNA in lumbar L4-L6 region of spinal cord from 2 and 12 days old rats, using Affymetrix Gene Chip and RT-PCR arrays. Fold change refers to the ratio of RNA expression in 12 day-old rats to 2 day-old rats ("-" indicates decrease). * Significant by the following 3 criteria: (1) fold change of "normalized" mean ≥ 1.5, by gene chip analyses (model-based dChip software); (2) fold change ≥ 1.2 by RT-PCR analyses; and (3) P value ≤ 0.05.

DETAILED DESCRIPTION

Neurotrophin-3 (NT-3) plays an important role in developing mammals. It acutely strengthens synaptic inputs to motoneurons in prenatal rats, an action that declines over the initial two postnatal weeks due to increased Mg^{2+} blockade of motoneuron N-methyl-D-aspartate receptors (NMDAR) required for this action of NT-3. This occurs concurrently with decreased expression of the NMDAR regulatory NR2D subunit in the spinal cord during the first postnatal week. Viral vector-mediated NR2D replacement in postnatal animals delays the age-related Mg^{2+} blockade and extends NT-3 action in postnatal animals. Thus, NR2D expression is an important link in converting NMDA-activated synapses from "active" to "silent", and the ability of NT-3 to strengthen synaptic transmission in neonatal motoneurons.

The invention is based, at least in part, on our investigation of the molecular bases of the age-dependent and synapse-selective motoneuron plasticity induced by NT-3, and on a demonstration of how this information can be used to extend the action of NT-3.

Compositions

While the compositions and methods of the present invention are not restricted to those that mediate any particular physiological mechanism, we believe they can be used to improve a sign or symptom associated with a disease or injury to the nervous

system by prolonging synaptic plasticity in neurons or restoring such plasticity after it is reduced or lost. These compositions (or "therapeutic agents," which may be directly administered or administered by way of expression vectors, include an NMDAR, subunits of NMDAR (*e.g.*, NR2D), neurotrophins (*e.g.*, NT-3), and various vehicles (*e.g.*, HSV-1 amplicon) for delivering the former compositions to targeted tissues and cells (*e.g.*, the types of neurons described herein).

NMDAR and Its Subunits

N-methyl-D-aspartate receptors (NMDAR) are protein receptors composed of a number of polypeptide subunits. These polypeptide subunits can include NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B. Many different combinations and permutations of these subunits are possible, and, consequently, there are many different subtypes of NMDARs (any of which can be encoded by the vectors and genetically modified cells of the present invention).

The polypeptide sequences of these NMDAR subunits, as well as the nucleotide sequences of the nucleic acids that encode them, are available in public sequence databases. In addition, much information is known about the structure and behavior of NMDAR and its subunits. For example, the characteristics of the NR2D subunit have been described by, among others, Wenzel *et al.* (*J. Neurochem.* 66(3):1240-1248, 1996) and Dunah *et al.* (*J. Neurochem.* 71(5):1926-1934), and its polypeptide sequence and the nucleotide sequence encoding this polypeptide are available, among other places, in SwissProt (polypeptide: Q03391), UniGene (nucleic acid: Hs.113286), and GenBank (both polypeptide and nucleic acid: L31611). This information is readily available to one of ordinary skill in the art and can be used to construct the vectors, cells, and pharmaceutical compositions described herein. NMDAR possess a number of binding sites, including a glutamate binding site and multiple sites to which modulatory compounds can bind. In addition, a number of NMDAR agonists (*e.g.*, glutamate, homoquinolinic acid), antagonists (*e.g.*, D-AP5, glycine site antagonists (*e.g.*, L-701,324, L-689,560, GV96771A), polyamine site antagonists (*e.g.*, Ro 25-6981), competitive antagonists (*e.g.*, (R)-AP5, (R)-CPP-ene, PBPD), and channel blockers (*e.g.*, MK-801, Memantine, Ketamine) have been described.

Neurotrophins

Neurotrophins are a family of neuronal growth factors that block apoptosis or neurons, promote nerve growth, and support neuronal differentiation, survival, and plasticity. Neurotrophins include nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4/5); these and other neurotrophins (as well as biologically active variants thereof) are useful in the present invention.

Neurotrophins are involved in both assisting neuronal survival early in development and maintaining neurons and neuronal function in the adult brain and peripheral nervous system. These growth factors also promote neuronal differentiation, neuronal stem cells, and neuronal outgrowth (Lewin and Barde, *Ann. Rev. Neurosci.* 19, 1996). Neurotrophins are also crucial in maintaining neuronal plasticity (Thoenen, *Science* 270:593-598, 1995). They also play important roles in recovery from nerve injury and in normal aging.

The structure and function of NT-3 have been elucidated. The nucleotide sequence of the nucleic acid encoding NT-3 is available in GenBank (*e.g.*, nucleic acid: M37763, M61180; polypeptide: AAA59953, AAA63231).

Methods of Administration

There are a variety of methods for successfully administering the compositions of the invention to a patient (*e.g.*, a patient amenable to treatment). These include any of the methods known for delivery of genes or genetic material to cells, including direct injection, high-speed bombardment (*e.g.*, by gene gun), and enhancing expression of the compositions using a cell's own genes. For example, one can use amplicons to enhance expression of NR2D in a patient's cells (see, for example, Example 6). However, other methods known in the art are also useful, including administration of the therapeutic agent (*i.e.*, the polypeptide) *per se*. To facilitate delivery of polypeptides or expression vectors to neuronal tissue, the tissue can be surgically exposed and the compositions of the invention can be applied to the exposed tissue. For example, physiologically acceptable compositions (*i.e.*, non-toxic compositions or those that provoke acceptable side effects) can be formulated as solutions, suspensions, gels, or the like, optionally placed within "slow release" devices, and applied to the exposed tissue (*e.g.*, tissue containing any of the types of neurons described herein in a patient amenable to treatment).

Amplicons

Helper virus-free systems for packaging herpesvirus particles, including those described herein, include at least one vector (herein, "the packaging vector") that, upon delivery to a cell that supports herpesvirus replication, will form a DNA segment (or segments) capable of expressing sufficient structural herpesvirus proteins that a herpesvirus particle will assemble within the cell. When the particle assembles, amplicon plasmids that may also be present, can be packaged within the particle as well. In packaging systems that employ helper viruses, amplicon plasmids rely on the helper virus function to provide the replication machinery and structural proteins necessary for packaging amplicon plasmid DNA into viral particles. Helper packaging function is usually provided by a replication-defective virus that lacks an essential viral regulatory gene. The final product of helper virus-based packaging contains a mixture of varying proportions of helper and amplicon virions. Recently, helper virus-free amplicon packaging methods were developed by providing a packaging-deficient helper virus genome via a set of five overlapping cosmids (Fraefel *et al.*, *J. Virol.* 70:7190-7197, 1996; see also U.S. Patent No. 5,998,208) or by using a bacterial artificial chromosome (BAC) that encodes for the entire HSV genome minus its cognate cleavage/packaging signals (Stavropoulos and Strathdee, *J. Virol.* 72:7137-7143, 1998; Saeki *et al.*, *Hum Gene Ther.* 9:2787-2794, 1998). Such cosmids can be used as the packaging vector in the methods described herein. Thus, the packaging vector can be a cosmid-based vector or a set of vectors including cosmid-based vectors that are prepared so that none of the viral particles used will contain a functional herpesvirus cleavage-packaging site containing sequence. This sequence, which is not encoded by the packaging vector(s), is referred to as the "*a*" sequence. The "*a*" sequence can be deleted from the packaging vector(s) by any of a variety of techniques practiced by those of ordinary skill in the art. For example, one can simply delete the entire sequence (by, for example, the techniques described in U.S. Patent No. 5,998,208). Alternatively, one can delete a sufficient portion of the sequence to render it incapable of packaging. Another alternative is to insert nucleotides into the site that render it non-functional.

The core of the herpesvirus particle is formed from a variety of structural genes that create the capsid matrix. It is necessary to have those genes for matrix formation present in a susceptible cell used to prepare particles. Preferably, the necessary envelope proteins are also expressed. In addition, there are a number of other proteins present on the surface of a herpesvirus particle. Some of these proteins help mediate viral entry into certain cells, and

as this is known to those of ordinary skill in the art, one would know to alter the sequences expressed by the viral particle in order to alter the cell type the viral particle infects or improve the efficiency with which the particle infects a natural cellular target. Thus, the inclusion or exclusion of the functional genes encoding proteins that mediate viral entry into cells will depend upon the particular use of the particle.

In addition to a packaging vector, the herpesvirus amplicon systems described herein include an amplicon plasmid. The amplicon plasmid contains a herpesvirus cleavage/packaging site containing sequence, an origin of DNA replication (ori) that is recognized by the herpesvirus DNA replication proteins and enzymes, and a transgene of interest (*e.g.*, a nucleic acid sequence that encodes a therapeutically effective protein). This vector permits packaging of desired nucleotide inserts in the absence of helper viruses. In some embodiments, the amplicon plasmid contains at least one heterologous DNA sequence that is operatively linked to a promoter sequence (we discuss promoter and other regulatory sequences further below). More specifically, the amplicon plasmid can contain one or more of the following elements: (1) an HSV-derived origin of DNA replication (ori) and packaging sequence ("*a*" sequence); (2) a transcription unit driven typically by the HSV-1 immediate early (IE) 4/5 promoter followed by an SV-40 polyadenylation site; and (3) a bacterial origin of replication and an antibiotic resistance gene for propagation in *E. coli* (Frenkel, *supra*; Spaete and Frenkel, *Cell* 30:295-304, 1982).

Amplicon plasmids are dependent upon helper virus function to provide the replication machinery and structural proteins necessary for packaging amplicon plasmid DNA into viral particles. Helper packaging function is usually provided by a replication-defective virus that lacks an essential viral regulatory gene. The final product of helper virus-based packaging contains a mixture of varying proportions of helper and amplicon virions. Recently, helper virus-free amplicon packaging methods were developed by providing a packaging-deficient helper virus genome via a set of five overlapping cosmids (Fraefel *et al.*, *J. Virol.* 70:7190-7197, 1996) or by using a bacterial artificial chromosome (BAC) that encodes for the entire HSV genome minus its cognate cleavage/packaging signals (Stavropoulos and Strathdee, *J. Virol.* 72:7137-7143, 1998; Saeki *et al.*, *Hum. Gene Ther.* 9:2787-2794, 1998).

Methods for generating helper virus-free Herpesvirus amplicons

Generally, the methods of the invention are carried out by transfecting a host cell with several vectors and then isolating HSV amplicon particles produced by the host cell (while the language used herein may commonly refer to a cell, it will be understood by those of ordinary skill in the art that the methods can be practiced using populations (whether substantially pure or not) of cells or cell types, examples of which are provided elsewhere in our description; such host cells are also within the scope of the present invention). The method for producing an hf-HSV amplicon particle can be carried out, for example, by co-transfecting a host cell with: (i) an amplicon vector comprising an HSV origin of replication, an HSV cleavage/packaging signal, and a heterologous transgene expressible in a cell; (ii) one or more vectors that, individually or collectively, encode all essential HSV genes but exclude all cleavage/packaging signals; and (iii) a vhs expression vector encoding a virion host shutoff protein. One can then isolate or purify (although absolute purity is not required; *e.g.*, the isolate may contain as little as 10% amplicon particles; greater purity may be preferred and isolates may be, *e.g.*, at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% pure amplicon particle) the HSV amplicon particles produced by the host cell. The therapeutic compositions of the invention can be similarly pure or unpure. When the HSV amplicon particles are harvested from the host cell medium, the amplicon particles may be referred to as "substantially pure" as they may be free of any other detectable virion particles, and present at a concentration of greater than about 1×10^6 (*e.g.*, about 2×10^6, 5×10^6, 1×10^7, 1×10^8, or more) particles per milliliter. To further enhance the use of the amplicon particles, the resulting stock can also be concentrated, which affords a stock of isolated HSV amplicon particles at a concentration of at least about 1×10^7 particles per milliliter. Such concentrated stocks are within the scope of the present invention and can be used in the methods described herein or packaged in the kits described herein.

The amplicon vector can either be in the form of a set of vectors or a single bacterial-artificial chromosome (" BAC"), which is formed, for example, by combining the set of vectors to create a single, doublestranded vector. As noted above, methods for preparing and using a five cosmid set are disclosed in, for example, Fraefel *et al.* (*J. Virol.*, 70:7190-7197, 1996), and methods for ligating the cosmids together to form a single BAC are disclosed in Stavropoulos and Strathdee (*J. Virol.* 72:7137-43, 1998).

The BAC described in Stavropoulos and Strathdee includes a *pac* cassette inserted at a *Bam*HI site located within the *UL41*coding sequence, thereby disrupting expression of the HSV-1 virion host shutoff protein.

By "essential HSV genes", it is intended that the one or more vectors include all genes that encode polypeptides that are necessary for replication of the amplicon vector and structural assembly of the amplicon particles. Thus, in the absence of such genes, the amplicon vector is not properly replicated and packaged within a capsid to form an amplicon particle capable of adsorption. Such "essential HSV genes" have previously been reported in review articles by Roizman (*Proc. Natl. Acad. Sci. USA* 11:307-1 13, 1996; *Acta Viroloeica* 43:75-80, 1999). Another source for identifying such essential genes is available at the Internet site operated by the Los Alamos National Laboratory, Bioscience Division, which reports the entire HSV-1 genome and includes a table identifying the essential HSV-1 genes. The genes currently identified as essential are listed in FIG. 3.

In other embodiments, a helper-free herpesvirus amplicon particle (*e.g.*, an hf-HSV) can be generated by: (1) providing a cell that has been stably transfected with a nucleic acid sequence that encodes an accessory protein (alternatively, a transiently transfected cell can be provided); and (2) transfecting the cell with (a) one or more packaging vectors that, individually or collectively, encode one or more (and up to all) HSV structural proteins but do not encode a functional herpesvirus cleavage/packaging site and (b) an amplicon plasmid comprising a sequence that encodes a functional herpesvirus cleavage/packaging site and a herpesvirus origin of DNA replication (ori). The amplicon plasmid described in (b) can also include a sequence that encodes a therapeutic agent. In another embodiment, the method comprises transfecting a cell with (a) one or more packaging vectors that, individually or collectively, encode one or more HSV structural proteins (*e.g.*, all HSV structural proteins) but do not encode a functional herpesvirus cleavage/packaging site, (b) an amplicon plasmid comprising a sequence that encodes a functional herpesvirus cleavage/packaging site, a herpesvirus origin of DNA replication, and a sequence that encodes an immunomodulatory protein (*e.g.*, an immunostimulatory protein), a tumor-specific antigen, an antigen of an infectious agent, or a therapeutic agent (*e.g.*, a growth factor), and (c) a nucleic acid sequence that encodes an accessory protein.

The HSV cleavage/packaging signal can be any cleavage/packaging that packages the vector into a particle that is capable of adsorbing to a cell (the cell being the target for

transformation). A suitable packaging signal is the HSV- I "a" segment located at approximately nucleotides 127- 1132 of the a sequence of the HSV- I virus or its equivalent (Davison *et al., J. Gen. Virol.* 55:315-331, 1981).

The HSV origin of replication can be any origin of replication that allows for replication of the amplicon vector in the host cell that is to be used for replication and packaging of the vector into HSV amplicon particles. A suitable origin of replication is the HSV- I "c" region, which contains the HSV- I ori segment located at approximately nucleotides 47-1066 of the HSV- I virus or its equivalent (McGeogh *et al., Nucl. Acids Res.* 14:1727-1745, 1986). Origin of replication signals from other related viruses (*e.g.*, HSV-2 and other herpes viruses, including those listed above) can also be used.

The amplicon plasmids can be prepared (in accordance with the requirements set out herein) by methods known in the art of molecular biology. Empty amplicon vectors can be modified by introducing, at an appropriate restriction site within the vector, a complete transgene (including coding and regulatory sequences). Alternatively, one can assemble only a coding sequence and ligate that sequence into an empty amplicon vector or one that already contains appropriate regulatory sequences (promoter, enhancer, polyadenylation signal, transcription terminator, *etc.*) positioned on either side of the coding sequence. Alternatively, when using the pHSVlac vector, the *LacZ* sequence can be excised using appropriate restriction enzymes and replaced with a coding sequence for the transgene. Conditions appropriate for restriction enzyme digests and DNA ligase reactions are well known in the art (*see, e.g.*, Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Laboratory, Cold Spring Harbor, New York (1989); Ausubel *et al.* (Eds.), *Current Protocols in -Molecular Biology*, John Wiley & Sons, New York, NY, 1999 and preceding editions; and U.S. Patent No. 4,237,224).

The amplicon systems featured in these methods and others described herein can all be modified so that the transgene carried by the amplicon plasmid is inserted into the genome of the host cell. Accordingly, the methods described herein can each include an additional step of introducing, into the host cell, a vector (which can be, but is not necessarily, a plasmid) that encodes an enzyme that mediates insertion of the transgene into the genome (this vector may be referred to herein as "an integration vector"). The integration vector can be applied to a host cell *in vivo* or in culture at the same time that one or more of the components of the amplicon system (*e.g.* the packaging vector or amplicon plasmid) are administered to the host cell. The enzyme

encoded by the integration vector can be a transposase, such as that encoded by *sleeping beauty* or a biologically active fragment or mutant thereof (*i.e.*, a fragment or mutant of the *sleeping beauty* sequence that facilitates integration of the transgene into the genome at a rate or to an extent that is comparable to that achieved when wild type *sleeping beauty* is used). As this system represents a fundamental advance over those in which the amplicon plasmid is maintained outside the genome (and is therefore "diluted out" as cells divide), it has broad application. Methods in which an integration vector is used in the context of an amplicon system, particularly including the hf-HSV systems described herein, can be carried out to treat patients with a wide variety of diseases or disorders associated with damage to nerves or neural cells (here, as in the methods described above, a "patient" is not limited to a human patient but can be any other type of mammal). For example, the patient can have damage to the spinal cord, Alzheimer's disease, or learning or memory deficiencies. Any of the specific types diseases or disorders involving nerve or neural cell damage (*e.g.*, spinal cord injury, Alzheimer's disease, learning or memory deficiencies) set out herein can be treated.

In addition, one can further modify the amplicon system (*i.e.*, any of the systems or compositions described herein, regardless of the precise therapeutic agent selected) to improve the safety of treatments in which an integration vector is administered. Frequent transposition events may lead to mutagenesis of the host genome and, possibly, even to proto-oncogene activation (although there is no evidence that this will occur or is likely to occur; it is speculated that the amplicon might enhance the frequency of such events, as 10-15 copies of the transgenon are present within a single virion). To regulate the transposase component of the system more tightly, one can, for example, incorporate the *Sleeping Beauty* protein into the virion in the form of a fusion with an HSV tegument protein. Alternatively, one could effect exogenous application of transposase protein with the transgenon-containing amplicon vector. Both approaches would prevent continued synthesis of *Sleeping Beauty* and thus, obviate additional catalysis of transposition. In yet another strategy, one could incorporate protein instability sequences into the open reading frame to limit transposase half-life. The transposon in the integration vector should be compatible with sequences flanking the transgene in the amplicon plasmid. For example, where the transposon is of the *Sleeping Beauty* system, the amplicon vector can include a transgene (for integration) flanked by the *Sleeping Beauty* terminal repeats. Integrating

forms of the HSV amplicon vector platform have been described previously. One form consists of an HSV amplicon backbone and adeno-associated virus (AAV) sequences required for integration.

The amplicon vector used in any of the methods described herein can also include a sequence that encodes a selectable marker and/or a sequence that encodes an antibiotic resistance gene. Selectable marker genes are known in the art and include, without limitation, galactokinase, beta-galactosidase, chloramphenicol acetyltransferase, beta lactamase, green fluorescent protein (GFP), alkaline phosphate, *etc.* Antibiotic resistance genes are also known in the art and include, without limitation, ampicillin, streptomycin, spectromycin, *etc.* A number of suitable empty amplicon vectors have previously been described in the art including, without limitation, pHSVIac (ATCC Accession 40544; U.S. Patent No. 5,501,979; Stavropoulos and Strathdee, *J. Virol.*, 72:7137-43, 1998), and pHENK (U.S. Patent No. 6,040,172). The pHSVIac vector includes the HSV-1 a segment, the HSV-1c region, an ampicillin resistance marker, and an *E. coli lacZ* marker. The pHENK vector includes the HSV-1 a segment, an HSV-1 ori segment, an ampicillin resistance marker, and an *E. coli LacZ* marker under control of the promoter region isolated from the rat preproenkephalin gene (*i.e.*, a promoter operable in brain cells). The sequences encoding a selectable marker, the sequences encoding the antibiotic resistance gene (which may also serve as a selectable marker), and the sequences encoding the transgene, may be under the control of regulatory sequences such as promoter elements that direct the initiation of transcription by RNA polymerase, enhancer elements, and suitable transcription terminators or polyadenylation signals. Preferably, the promoter elements are operable in the cells of the patient that are targeted for transformation. A number of promoters have been identified that are capable of regulating expression within a broad range of cell types. These include, without limitation, HSV immediate-early 4/5 (IE4/5) promoter, cytomegalovirus ("CMV") promoter, SV40 promoter, and P-actin promoter. Likewise, a number of other promoters have been identified that can regulate expression within a narrow range of cell types. These include, without limitation, the neural-specific enolase (NSE) promoter, the tyrosine hydroxylase (TH) promoter, the GFAP promoter, the preproenkephalin (PPE) promoter, the myosin heavy chain (MHQ promoter), the insulin promoter, the cholineacetyltransferase (ChAT) promoter, the dopamine β-hydroxylase (DBH) promoter, the calmodulin dependent kinase (CamK)

promoter, the c-fos promoter, the c-jun promoter, the vascular endothelial growth factor (VEGF) promoter, the erythropoietin (EPO) promoter, and the EGR- I promoter. The transcription termination signal should, likewise, be operable in the cells of the patient that are targeted for transformation. Suitable transcription termination signals include, without limitation, polyA signals of HSV genes such as the vhs polyadenylation signal, SV40 poly-A signal, and CW IE1 polyA signal.

Applying the information above in effective gene therapies for neural damage has been hampered by the lack of a safe and reliable vector that can be used to transduce nerve cells. Nerve cells are effectively post-mitotic. Although both retroviral and adenoviral vectors have been employed in different clinical trials for gene therapy, both systems exhibit limitations (Uckert and Walther, *Pharmacol. Ther.* 63:323-347, 1994; Vile *et al.*, *Mol. Biotechnol.* 5:139-158, 1996; Collins, *Ernst Schering Research Foundation Workshop*, 2000; Hitt *et al.*, *Adv. Pharmacol.* 40:137-206, 1997; Kochanek, *Hum. Gene Ther.* 10:2451-2459, 1999). For example, the low levels of integrin receptors for adenovirus on CLL cells mandates the use of very high adenovirus titers, preactivation of the CLL cell with IL-4 and/or anti-CD40/CD40L (Cantwell *et al.*, *Blood* 88:4676-4683, 1996; Huang *et al.*, *Gene Ther.* 4:1093-1099, 1997), or adenovirus modification with polycations to achieve clinically meaningful levels of transgene expression (Howard *et al.*, *Leukemia* 13:1608-1616, 1999).

HSV amplicon particles can be used to transduce nerve cells (e.g., mouse, rat, human, or other mammalian). Vectors can be constructed to encode β-galactosidase (by inclusion of the *lacZ* gene), B7.1 (also known as CD80), or CD40L (also known as CD154), and they can be packaged using either a standard helper virus (HSVlac, HSVB7.1, and HSVCD40L) or by a helper virus-free method (hf-HSVlac, hf-HSVB7.1, and hf-HSVCD40L). Cells transduced with these vectors have been studied for their expression of heterologous genes. High rates of expression in these studies have indicated that this means of gene therapy is an efficacious and reliable means of delivering heterologous genes. These studies support the conclusion that HSV amplicons are efficient vectors for gene therapy, particularly of neurons, and that helper virus-free amplicon preparations are well suited for use in therapeutic compositions.

Therapeutic Agents

As noted, the hf-HSV amplicon particles described herein (and the cells that contain them) can express a heterologous protein (*i.e.*, a full-length protein or a portion thereof (*e.g.*, a functional domain or antigenic peptide) that is not naturally encoded by a herpesvirus). The heterologous protein can be any protein that conveys a therapeutic benefit on the cells in which it, by way of infection with an hf-HSV amplicon particle, is expressed or a patient who is treated with those cells.

When used for gene therapy, the transgene encodes a therapeutic transgene product, which can be either a protein or an RNA molecule.

Therapeutic RNA molecules include, without limitation, antisense RNA, inhibitory RNA (siRNA), and an RNA ribozyme. The RNA ribozyme can be either *cis* or *trans* acting, either modifying the RNA transcript of the transgene to afford a functional RNA molecule or modifying another nucleic acid molecule. Exemplary RNA molecules include, without limitation, antisense RNA, ribozymes, or siRNA to nucleic acids for huntingtin, alpha synuclein, scatter factor, amyloid precursor protein, p53, VEGF, *etc.*.

Therapeutic proteins include, without limitation, NMDAR or any subunit thereof, such as NR1, NR2A, NR2B, NR2C, NR2D, NR3A, or NR3B (as noted above, any of the compositions of the present invention, or methods in which they are used, can include biologically active (*e.g.*, therapeutically useful) variants (*e.g.*, substitution, deletion, or addition mutants) of NMDARs, neurotrophins, or other therapeutic proteins (*e.g.*, a neurotrophin such as NT-3 or a biologically active variant thereof).

Formulation and Administration of hf-HSV amplicon particles

The hf-HSV amplicon particles described herein can be administered to patients directly or indirectly; alone or in combination with other therapeutic agents; and by any route of administration. For example, the hf-HSV amplicon particles can be administered to a patient indirectly by administering cells transduced with the vector to the patient. Alternatively, or in addition, an hf-HSV amplicon particle (or any other HSV amplicon particle) could be administered directly. For example, an hf-HSV amplicon particle that expresses an NR2D protein can be introduced into spinal cord tissue by, for example, introducing the vector into the tissue or into the vicinity of the tissue.

Administration of HSV protein amplicons encoding NMDAR or its subunits (*e.g.*, NR2D) provide therapeutic benefits, we believe, in the form of restoration of neural

plasticity and nerve function. The helper virus-free HSV vectors disclosed herein can be administered in the same manner.

The herpesvirus amplicon particles described herein, and cells that contain them, can be administered, directly or indirectly, with other species of HSV-transduced cells (*e.g.*, HSV-NR2D transduced cells) or in combination with other therapies. Such administrations may be concurrent or they may be done sequentially. Thus, in one embodiment, HSV amplicon particles, the vectors with which they are made (*i.e.*, packaging vectors, amplicon plasmids, and vectors that express an accessory protein) can be injected into a living organism or patient (*e.g.*, a human patient) to treat, for example, spinal cord damage or Alzheimer's disease. In further embodiments, one or more of these entities can be administered after administration of a therapeutically effective amount of another substance.

The concentrated stock of HSV amplicon particles is effectively a composition of the HSV amplicon particles in a suitable carrier. HSV amplicon particles can also be administered in injectable dosages by dissolving, suspending, or emulsifying them in physiologically acceptable diluents with a pharmaceutical carrier (at, for example, about 1×10^7 amplicon particles per ml). Such carriers include sterile liquids, such as water and oils, with or without the addition of a surfactant and other pharmaceutically and physiologically acceptable carriers, including adjuvants, excipients or stabilizers. The oils that can be used include those obtained from animals or vegetables, petroleum based oils and synthetic oils. For example, the oil can be a peanut, soybean, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, glycols (*e.g.*, propylene glycol or polyethylene glycol) are preferred liquid carriers, particular when the amplicon particles are formulated for administration by injection.

For use as aerosols, the HSV amplicon particles, in solution or suspension, can be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutene with conventional adjuvants. The particles can also be administered in a non-pressurized form such as in a nebulizer or atomizer.

Other Methods of Administration

In addition to gene therapy (*e.g.*, using hf-HSV amplicons), the invention also includes administration of NMDAR, subunits of NMDAR (*e.g.*, NR2D), or

neurotrophins (*e.g.*, NT-3) by other methods. These methods include direct injection of amplicon particles, nucleic acids or the polypeptides they encode into a target tissue or a fluid that contacts the target tissue (*e.g.*, where the target tissue is within the CNS, the amplicon particle can be injected into cerebrospinal fluid), introduction of cells transduced by a nucleic acid or polypeptide of interest into target tissue (or, similarly, a fluid that contacts the target tissue), bombardment at high velocity of target tissue with amplicon particles, nucleic acids or polypeptides of interest, enhancing endogenous expression of one or more of the polypeptides of interest, as well as various other methods known to those of skill in the art. These methods are united by the result: delivery of therapeutically effective amounts of NMDAR, subunits of NMDAR (*e.g.*, NR2D), or neurotrophins (*e.g.*, NT-3) to a targeted tissue (*e.g.*, damaged spinal cord, brain, or other neural tissue).

Methods of Treatment

Patients Amenable To Treatment

As noted above, the methods of the invention can be employed to treat a variety of patients, including those who have been diagnosed as having a compromised nervous system or who are at risk for developing a neural deficit (which may manifest as a motor, sensory, or cognitive deficit, or any combination thereof). For example, the patient may have experienced, or be expected to experience, a traumatic injury. While the precise cause of the injury is irrelevant, we note that it may be, for example, a sporting event such as boxing, gymnastics, or horseback riding; an automobile, boating, biking, or airplane accident; an accident at a construction site or industrial facility; or a medical or surgical procedure (*e.g.*, excision of a brain tumor or other neurosurgery, including surgery on or around the spinal cord). The cells harmed by the injury may include neurons that naturally express an NMDA receptor and/or neurotrophin receptors (*e.g.*, receptors for NT-3). For example, they may include populations of neurons that constitute the motor compartment (*e.g.*, anterior horn motoneurons) as well as those that project through the spinal cord and whose cell somata lie elsewhere (*e.g.*, neurons of the corticospinal tract, rubrospinal tract, and the like). While we focus here on traumatic injury, patients with a disease that affects these types of neurons are also amenable to treatment with the methods described herein.

Accordingly, other patients amenable to treatment may be suffering from a disease that causes a sensory, motor, or cognitive deficit (we use the term "disease" broadly to refer to a state of ill health; the precise condition may be one that is commonly referred to as a disorder, syndrome, or the like). For example, patients amenable to treatment may be diagnosed as having Alpers' Disease (a rare, genetic disease that causes progressive brain degeneration; this disease often manifests as seizures in young children), Alzheimer's Disease, cerebral palsy, corticobasal ganglionic degeneration (CBD; another rare and progressive neurological disorder; characterized by Parkinsonism and cortical dysfunction) and the related condition, progressive supranuclear palsy (PSP), Freidreich's ataxia (which features degeneration of the spinal cord, the cerebellum, and sensory nerves to the hands and feet), Huntington's Disease, Lewy body dementia (LBD; characterized by fluctuating cognition, recurrent visual hallucinations, and Parkinsonism), multiple system atrophy (MSA, a term synonymous with striatonigral degeneration (SND) when Parkinsonism predominates; with olivopontocerebellar atrophy (OPCA) when cerebellar signs predominate; and with Shy-Drager syndrome when autonomic failure is dominant), Parkinson's Disease, an idiopathic acquired ataxia such as olivopontocerebellar atrophy, postpoliomyelitis syndrome (as the name implies, progressive weakness in previously affected muscles of an individual who had poliomyelitis at least 10 years earlier), a prion disease (*e.g.*, Creutzfeldt-Jakob Disease (CJD)), Rett syndrome, and tuberous sclerosis. Other patients may have a motor neuron disease such as amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) or spinal muscular atrophy (SMA). Where ALS is hereditary, it may be caused by a dominant, recessive, or X-linked mutation. Regardless of the underlying genetic defect, a patient diagnosed with ALS is amenable to treatment with the compositions and methods described herein. There are various types of SMA, which differ in their time of onset and severity. Type I SMA, also called Werdnig-Hoffmann Disease, is the most severe and is recognized in many cases before three months of age. Type II (or chronic) SMA is usually diagnosed before the patient is two years old, and the mildest form (Type III, also known as Kugelberg-Welander or Juvenile Spinal Muscular Atrophy), is usually diagnosed between about 18 months of age and adolescence. Children with Type III SMA can stand alone and walk with difficulty. Type IV SMA develops in adulthood,

typically after the age of 35. Where the disease is X-linked, it may be referred to as Kennedy's Syndrome or bulbo-SMA.

Other patients amenable to treatment may have experienced nerve damage due to a birth defect such as hydrocephalus or spina bifida. The nervous system may also have been damaged by a traumatic birth or a surgical procedure thereafter (*e.g.*, a surgical procedure to separate conjoined twins).

Yet other amenable patients are those having an age-related spinal myelopathy (*e.g.*, a cervical myelopathy) or any other condition in which neuronal function is compromised by compression.

Delivery To Target Tissue

The compositions of the present invention (including amplicons that express an NMDAR, subunits of NMDAR (*e.g.*, NR2D), and/or a neurotrophin (*e.g.*, NT-3), herpesvirus particles, and cells that contain them) can be used to restore or maintain neural synaptic plasticity in patients who have experienced neural damage or loss of synaptic plasticity. A patient can be treated after they have been diagnosed with neural damage. Alternatively, the compositions of the invention can be used to treat patients before neural damage (or before substantial neural damage) has occurred. Thus, "treatment" can encompass prophylactic treatment (in which case the patient experiences, for example, a reduced risk of developing a neural deficit or may develop a deficit that is less severe in some way than it would have been in the absence of treatment (*e.g.*, less rapid in onset or less profound). Patients considered to have an elevated risk of developing a neural deficit may be identified by genetic screening (*e.g.*, patients who carry expanded poly-glutamine repeats in the *huntingtin* gene). The methods of the invention can include a step of identifying a patient having an elevated risk of a neural deficit by, for example, genetic screening and/or family history.

HSV amplicon particles have been used to transduce motoneurons. The vectors can be constructed to encode β-galactosidase (by inclusion of the *lacZ* gene) and NR2D, and they can be packaged using either a standard helper virus (*e.g.*, HSVlac, HSVB7.1, and HSVCD40L) or by a helper virus-free method (*e.g.*, hf-HSVlac, hf-HSVB7.1, and hf-HSVCD40L). Motoneurons transduced with these vectors were studied for their synaptic plasticity. It has been demonstrated that synaptic plasticity can be prolonged past birth in a mammalian model. These studies support the

conclusion that HSV amplicons are efficient vectors for gene therapy, and that helper virus-free amplicon preparations are well suited for use in therapeutic compositions.

Formulation and Administration of hf-HSV amplicon particles

The hf-HSV amplicon particles described herein can be administered to patients directly or indirectly; alone or in combination with other therapeutic agents; and by any route of administration. For example, the hf-HSV amplicon particles can be administered to a patient indirectly by administering cells transduced with the vector to the patient. Alternatively, or in addition, an hf-HSV amplicon particle could be administered directly. For example, an hf-HSV amplicon particle that expresses an NMDAR protein or subunit (*e.g.*, NR2D) can be introduced into target nerve tissue by, for example, injecting the vector into the nerve tissue or into the vicinity of the nerve tissue.While the compositions of the invention are not limited to those that exert a therapeutic benefit by any particular mechanism of action, we believe that administration of HSV amplicons encoding NMDAR or its subunits (*e.g.*, NR2D) maintain or restore neural synaptic plasticity. The herpesvirus amplicon particles described herein, and cells that contain them, can be administered, directly or indirectly, with other species of HSV-transduced cells (*e.g.*, cells transduced with immunomodulatory agents) or in combination with other therapies. Such administrations may be concurrent or they may be done sequentially. Thus, in one embodiment, HSV amplicon particles, the vectors with which they are made (*i.e.*, packaging vectors, amplicon plasmids, and vectors that express an accessory protein) can be injected into a living organism or patient (*e.g.*, a human patient) to treat, for example, a spinal cord injury or Alzheimer's disease. In further embodiments, one or more of these entities can be administered after administration of another therapeutically effective composition.

Enhanced Delivery Via Surgery

Surgical techniques can be employed to enhance delivery of the therapeutic compositions of the invention by allowing close access to target tissue. For example, careful dissection can be used to separate and retract the skin and muscles overlying damaged spinal cord tissue, and the underlying spinal cord segment exposed. Viral particles, such as the amplicon particles described herein, can be introduced (*e.g.*, by

direct injection) directly into the ventral horn (*e.g.*, two injections in a small volume (*e.g.*, 0.1 – 5.0 (*e.g.* 1.0) µl). The muscle and skin can then be sutured, the skin stitched or glued using surgical skin glue, and the surgical entry wound properly treated with antibiotics and other necessary medicaments. Such surgical techniques are well known to those of skill in the surgical arts.

Testing For Successful Treatment

After treatment using the compositions or methods of the invention, it is possible to test treated patients to assess treatment success. One of skill in the neurological arts would be well aware of the appropriate tests to measure treatment success (*e.g.*, tests of balance, fine motor skill, and cognition). For example, a patient treated for a spinal cord injury can be assessed using standard neurological tests of spinal cord function. Similarly, a patient treated for Alzheimer's disease can be assessed using standard cognitive tests of brain function (*e.g.*, learning and memory). In addition, high-definition imaging techniques (*e.g.*, MRI) can be used to assess directly neural response to treatment.

Kits

The invention includes kits that can be used to maintain or increase neuronal plasticity, strengthen synaptic transmission, and improve memory or learning. These kits can include all of the necessary reagents for carrying out the methods of the invention, and can include any of the compositions of the invention. In addition, kits can include detailed instructions for effective use. For example, a kit for treating spinal cord injuries can include amplicons containing NR2D and NT-3, detailed instructions for administering the amplicons to the appropriate tissue, and instructions for confirming the effectiveness of amplicon therapy.

EXAMPLES

EXPERIMENTAL METHODS

Electrophysiology

Intracellular recordings (microelectrodes 70-110 MW filled with 3 M potassium acetate) were obtained from antidromically identified lumbar spinal motoneurons in the L5 region in 67 spinal cord preparations isolated from halothane-anaesthetized

Sprague-Dawley rats 1-5 or 8-15 days old, or at embryonic day 18 (E18; Pinco and Lev-Tov, *J. Neurophysiol.* 70:406, 1993; Arvanov *et al.*, *J. Neurophysiol.* 84:752, 2000). The left hemicord was superfused with ACSF containing (in mM): NaCl (117), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (2.0), $NaHCO_3$ (25), NaH_2PO_4 (1.2), dextrose (Arvanov *et al.*, *J. Neurophysiol.* 84:752, 2000), aerated with 95% O_2 / 5% CO_2 (pH 7.4, 30° C) at10 ml/min. The VLF was dissected free of the spinal cord at T2 (Pinco and Lev-Tov, *J. Neurophysiol.* 72:2406 (1994); Arvanov *et al.*, *J. Neurophysiol.* 84:752, 2000). Suction stimulating electrodes were attached to the peeled VLF axon bundles and to the cut L5 dorsal root for activation of inputs to motoneurons, and to the L5 ventral root for antidromic activation of motoneurons. Electrical stimulation of the DR and/or VLF was at a rate of 0.01 Hz, 60 ms duration at an intensity just-maximum for evoking the monosynaptic potential in normal ACSF (about 100 mA for DR and 500 mA for VLF). The AMPA/kainate receptor antagonist CNQX (10 mM), GABAA receptor antagonist bicuculline (5 mM), GABAB receptor antagonist CGP 35348 (10 mM) and glycine receptor antagonist strychnine (5 mM) were added to the perfusion solution to isolate NMDAR-mediated responses pharmacologically. In the experiments with reduced concentrations of Mg^{2+}, corresponding equiosmolar changes in Na^+ concentration were made. All cells included in this study displayed a resting membrane potential of –64 mV to –67 mV.

Gene Chip Array

To compare the gene expression in L4-L6 region of rat spinal cord in 2 and 12 days old rats, Affymetrix (Santa Clara, CA) oligonucleotide arrays (Zhao *et al.*, *J. Comp. Neurol.* 441:187, 2001) were employed. Each group was represented by two replicate samples, using five L4-L6 sections of dissected rat spinal cord per sample and two microarrays. All procedures were performed at the CRPF/Salk Functional Genomic Laboratory in accordance with the instructions provided by Affymetrix. Total RNA (10 μg) was converted into double-stranded cDNA by using an oligo-dT primer with a T7 promoter (Life Technologies, Inc.). Double-stranded cDNA was extracted with phenol/chloroform and used for *in vitro* transcription with a T7 RNA polymerase. The labeled cRNA was purified with RNeasy™ columns. After calculating the cRNA concentration by using a 260A spectrometer, cRNA was fragmented, and used to prepare hybridization cocktail containing oligonucleotides. The oligonucleotides were

hybridized and the hybridization fluorescent results captured with Affymetrix equipment and protocols. To correct for minor differences in overall chip fluorescence, data from each microarray were normalized (Zhao *et al., J. Comp. Neurol.* 441:187, 2001). For analyzing the scanned data and calculations of gene expression levels we used GeneChip (version 4.1, Affymetrix) and model-based dChip (Li and Wong, *Genome Biol.* 2:1, 2001) software. It has been established that a change of expression level of 1.8-fold or greater (GeneChip analyses), or 1.2 or greater with p value of 0.05 or less (dChip analyses), are significant, accurate and biologically meaningful (Zhao *et al., J. Comp. Neurol.* 441:187, 2001; Li and Wong, *Genome Biol.* 2:1, 2001). In this study, a stricter cut-off was used for selecting genes that are developmentally regulated: a fold change (of the "normalized" mean) of at least 1.5 with $p \leq 0.05$ scored by dChip software.

Real Time Quantitative RT-PCR Array

Changes in gene expression level in L4-L6 region of rat spinal cord in 2 and 12 days old rats were confirmed by real-time reverse transcription and polymerase chain reaction (real-time RT-PCR). One microgram of total RNA was reverse transcribed using random hexamer priming and AMV reverse transcriptase according to the manufacturer's protocol (Invitrogen). cDNA pools were diluted 1:5 and 2.5 µl of these diluted samples were analyzed in a standard PE7700 quantitative PCR reaction using a designed NR1 or NR2D-specific primer/probe combination multiplexed with an 18S rRNA-specific primer/probe set (internal control). The NR1 probe sequence was 5'-CGCTGCCACAGTGTACCGCGC-3' (SEQ ID NO:1); the NR1 sense primer sequence was 5'-TCCTTTCTGCAAGCGAGGAC-3' (SEQ ID NO:2); and the NR1 antisense primer sequence was 5'-GCCCGTCATGTTCAGCATT-3' (SEQ ID NO:3). The NR2D probe sequence was 5'-CCTGCAGCTTGGCTCCTCCACAG-3' (SEQ ID NO:4); the NR2D sense primer sequence was 5'-GTGCTCACACCCAAGGAGAAG-3' (SEQ ID NO:5); and the NR2D antisense primer sequence was 5'-CCTCAAAAATGACCTGCAACTG-3' (SEQ ID NO:6). The 18S rRNA probe sequence was 5'-TGCTGGCACCAGACTTGCCCTC-3' (SEQ ID NO:7); the 18S rRNA sense primer sequence was 5'-CGGCTACCACATCCAAGGAA-3' (SEQ ID NO:8); and the 18S rRNA antisense primer sequence was 5'-GCTGGAATTACCGCGGCT-3' (SEQ ID NO:9). Each 25-µl PCR sample contained 2.5 µl of diluted cDNA pool, 900 nM of each primer, 50 nM of each probe,

and 12.5 µl of 2X Perkin-Elmer Master Mix (PE-Applied Biosystems). Following a 2-minute, 50°C incubation, and a 2-minute 95°C denaturation step, the samples were subjected to 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescent intensity of each sample was detected automatically during the cycles by the Perkin-Elmer Applied Biosystem Sequence Detector 7700 machine. Each PCR run included the following: no-template control samples, "no-reverse transcriptase" controls, and standard curve dilution series for each of the target sequences. Following the PCR run, "real-time" data were analyzed using Perkin-Elmer Sequence Detector Software version 1.6.3 and regression analyses were performed to calculate relative levels of NR1 or NR2D transcript in each test sample.

HSV Amplicon Vector Construction And Packaging

Helper virus-free amplicon packaging and virus purification was performed as previously described (Bowers *et al.*, *Mol. Ther.* 1:294, 2000; Bowers *et al.*, *Gene Ther.* 8:111, 2001). Amplicon virus numbers were determined by assessing both expression and transduction titers as previously described (Bowers *et al.*, *Mol. Ther.* 1:294, 2000; Bowers *et al.*, *Gene Ther.* 8:111, 2001).

Implantation Of Amplicons Expressing Viral Particles

Implantation was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at SUNY-Stony Brook. Two-day old Sprague-Dawley rats were anesthetized by hypothermia by placing them on a latex glove in contact with a bed of ice for 10 minutes. Under a dissecting microscope the skin and muscles overlying the lumbar spinal cord at L4-L6 were separated and retracted, and the underlying spinal cord was exposed. Transduction units (~10^4 viral particles) were injected directly into L4 and L6 ventral horns (2 injections of 1 µl each) by using sharp glass micropipette. The muscle and skin were sutured in layers with 5-0 silk sutures. Finally, the skin was glued using Vetbond™ (3M Corp.) tissue adhesive, and the wound was covered with sesame oil to prevent the mother from rejecting the pup. Pups were kept warm and were returned to the mother when they became active. After 8-10 days of treatment, the rats were prepared for intracellular recordings.

STUDIES

Example 1. Effects Of NT-3 On Synaptic Responses Elicited By DR And VLF

A prior study demonstrated the necessity for NMDA receptor function for acute neurotrophin-induced alteration of monosynaptic transmission at resting membrane potential in neonatal rats (Arvanov *et al.*, *J. Neurophysiol.* 84:752, 2000; Arvanian and Mendell, *Eur. J. Neurosci.* 14:1800, 2001; Arvanian and Mendell, *J. Neurophysiol.* 86:123, 2001). An important determinant of NMDA receptor function is its susceptibility to Mg^{2+} block at resting membrane potential (Nowak *et al.*, *Nature* 307:462, 1984). We evoked monosynaptic responses separately by stimulating DR and VLF and recorded the responses as a function of different extracellular [Mg^{2+}]. These experiments were performed initially in rats of two age groups, 1-5 and 8-15 day old (d.o.).

As before, DR- and VLF-evoked responses in "normal" (Arvano *et al.*, *J. Neurophysiol.* 84:752, 2000); see EXPERIMENTAL METHODS) saline (dotted traces in FIG. 1A) were recorded; then all other known inputs to the motoneuron were blocked pharmacologically in order to isolate the NMDAR-mediated response. In a solution containing 100 μM and lower concentrations of Mg^{2+}, the peak amplitude of the monosynaptic response (arrowhead in FIG. 1A) for both DR and VLF stimulation did not change over this immediate postnatal period [DR: 1-5 d.o. 7.4±0.5 mV (n=8), 8-15 d.o. 6.6±0.7 mV (n=6); VLF: 1-5 d.o. 5.7±0.4 mV (n=8), 8-15 d.o. 6.1±0.7 mV (n=6); FIGs. 1A and 1B]. However, increases in [Mg^{2+}] induced a decline of NMDAR-mediated VLF-responses at lower Mg^{2+} concentrations than DR-responses in the same motoneuron. The calculated dissociation constant (Chen and Huang, *Nature* 356:521, 1992) for the Mg^{2+} block (KMg) of the response to DR stimulation (1199 μM, n=8) was about 3 times larger than KMg for the response evoked by VLF stimulation (428 μM, n=8) in the same population of motoneurons recorded from 1-5 d.o. rats (FIG. 1B). The dissociation constant for the Mg^{2+} block of NMDAR-mediated DR-responses decreased nearly 50% between 1-5 and 8-15 d.o. animals (KMg = 691 μM; FIGs. 1A and 1B), while the KMg for NMDA-mediated VLF-responses in 8-15 d.o. rats (430 μM, n=6) was not different from that of 1-5 d.o. animals. Thus, at resting membrane potential and in physiological Mg^{2+} concentrations (1-2 mM), NMDAR-mediated responses of individual motoneurons at descending VLF connections exhibited a

markedly higher sensitivity to extracellular Mg^{2+} than NMDAR-mediated responses of the same motoneuron at the segmental DR connections.

Example 2. Mg^{2+} Sensitivity Of The VLF Response

To investigate whether the Mg^{2+} sensitivity of the VLF response which was constant throughout the first 2 postnatal weeks might exhibit reduced sensitivity to Mg^{2+} at an earlier stage of development, synaptic responses at E18, soon after synaptic connections to motoneurons begin to function (Ziskind-Conhaim, *J. Neurosci.* 10:125, 1990) were examined. Note that in the presence of 2 mM Mg^{2+}, administration of antagonists to AMPA/kainate, GABAA, B and glycine receptors induced only minor changes in the amplitude of monosynaptic responses at both DR and VLF inputs to E18 motoneurons (FIG. 1A), while these antagonists blocked the initial monosynaptic component in 1 and 2 w.o. animals (FIG. 1A). These results suggest that NMDA receptors mediate a large fraction of the synaptic input to motoneurons of prenatal rats, which is consistent with a previous observation of Ziskind-Conhaim (Ziskind-Conhaim, *J. Neurosci.* 10:125, 1990). While comparing the dependence of NMDA receptor-mediated responses on extracellular Mg^{2+}, it was found that the VLF response was considerably less sensitive to Mg^{2+} at E18 (K_{Mg}= 989 μM) than in the first postnatal week (K_{Mg}= 428 μM), and the DR response, already quite insensitive during the first postnatal week (K_{Mg}= 1199 μM) was even less sensitive at E18 (K_{Mg}= 1856 μM). Thus, it appears that the NMDA receptors on motoneurons associated with DR input change continuously from E18 into the second postnatal week whereas those associated with VLF input reach a plateau during the first postnatal week.

Example 3. NT-3 Potentiation Of The Response To Stimulation Of VLF At E18

It was then investigated whether NT-3 potentiates the response to stimulation of VLF at E18, in which the degree Mg^{2+} blockade of the associated NMDAR is normally relatively small. NT-3 facilitated both DR- and VLF-evoked monosynaptic responses in motoneurons of E18 prenatal rats (FIGs. 1D and 1E) in contrast to its effect in neonatal rats where during the first week it potentiated inputs from DR but not VLF, and the second week where neither input was potentiated by NT-3 (Arvanov *et al.*, *J. Neurophysiol.* 84:752, 2000; Arvanian and Mendell, *Eur. J. Neurosci.* 14:1800, 2001; Arvanian and Mendell, *J. Neurophysiol.* 86:123, 2001). These changes in NT-3 effects

are consistent with the decline of function of NMDAR associated with these synaptic inputs.

Example 4. Mg^{2+} Dependence Of AMPA/kainate Monosynaptic Responses

In addition to unblocking NMDA receptors, reducing extracellular Mg^{2+} increases presynaptic release of glutamate that may contribute to enhancement of the synaptic response (Kuno and Takahashi, *J. Physiol.* 376:543, 1986). This possible explanation of FIG. 1B was evaluated by comparing the Mg^{2+} dependence of AMPA/kainate monosynaptic responses evoked by stimulation of DR and VLF during the first postnatal week. FIG. 1C illustrates that the decline of the AMPA/kainate receptor-mediated response elicited by VLF and DR with increasing Mg^{2+} was similar and considerably less than the decline in the NMDAR-mediated response during the same period. These findings are consistent with previous reports that elevating Ca^{2+} concentration in the presence of 2 mM Mg^{2+} (2mM Mg^{2+}/ 4 mM Ca^{2+}), known to enhance presynaptic release of glutamate without affecting NMDAR Mg^{2+} block, did not cause the appearance of VLF-NMDAR-mediated responses in 1 or 2 week-old animals (Arvanian and Mendell, *J. Neurophysiol.* 86:123, 2001). Taken together, these results suggest that the differential Mg^{2+} dependence of NMDAR-mediated responses at the DR and VLF connections in 1-5 and 8-15 day-old animals is determined mainly by the magnitude of the Mg^{2+} block of NMDA receptors, rather than by the level of released glutamate.

Example 5. Molecular Mechanisms Of Age-Dependent Changes In Mg^{2+} Blockade

The degree of Mg^{2+} blockade of the NMDA receptor is dependent upon its subunit composition (Monyer *et al.*, *Neuron* 12:529, 1994; Burnashev *et al.*, *J. Physiol.* 485:403,1995). NMDARs are composed of NR1, NR2A-D and NMDAR-L (NR3A,B) subunits (Sucher *et al.*, *J. Neurosci.* 15:6509, 1995; Abdrachmanova *et al.*, *J. Physiol.* 538:53, 2002; Cull-Candy *et al.*, *Curr. Opin. Neurobiol.* 11:327, 2001). Somata and dendrites of neonatal rat motoneurons express these subunits (Abdrachmanova *et al.*, *J. Physiol.* 538:53, 2002; Virgo *et al.*, *J. Neuropathol. Appl. Neurobiol.* 26:258, 2000). NR1 subunits are essential for the formation of functional NMDAR, whereas other subunits modify receptor properties, including Mg^{2+} block (Burnashev *et al.*, *J. Physiol.*

485:403, 1995; Momiyama *et al.*, *J. Physiol.* 494:479, 1996). To address possible molecular mechanisms of age-dependent changes in the degree of Mg^{2+} blockade and corresponding decrease in NT-3 induced plasticity, it was examined whether the elevated level of Mg^{2+} blockade of NMDAR in 2 week-old animals was associated with a change in NMDAR subunit composition. Initially, the Affymetrix array was used to compare mRNA content in the L4-L6 spinal cord region between 2 and 12 day-old rats. Of 8,728 genes assayed, 246 showed statistically significant changes in mRNA expression (as determined by criteria enumerated in EXPERIMENTAL METHODS). Among these was the transcript encoding the NR2D subunit, which decreased from the first to the second postnatal week. The expression of other NMDAR subunits did not exhibit statistically significant changes during the first two postnatal weeks (FIG. 4). Real time quantitative RT-PCR analyses confirmed the age-related decrease in the level of NR2D subunit mRNA expression during the first two postnatal weeks and no significant changes in NR1 transcript levels (FIG. 4).

Example 6. Up-Regulation Of NR2D Subunit Expression And Delay Of Mg^{2+} Blockade

Considering that the presence of NR2D subunits makes the NMDA receptor resistant to Mg^{2+} blockade (Momiyama *et al.*, *J. Physiol.* 494:479, 1996), we hypothesized that the decreased expression of NR2D subunits results in the development of NMDAR Mg^{2+} blockade and the decay of NT-3 neurotrophic effects during the first postnatal week. To test this hypothesis, we next examined whether up-regulation of NR2D subunit expression would delay development of Mg^{2+} blockade at both synaptic inputs and reestablish the NT-3 induced motoneuron plasticity. The Herpes Simplex virus type-1 (HSV-1) amplicon (Bowers *et al.*, *Mol. Ther.* 1:294, 2000; Bowers *et al.*, *Gene Ther.* 8:111, 2001) was employed to deliver either NR2D (HSVnr2d) or *E. coli*-derived ß-galactosidase as a control (HSVlac) by direct injections into the ventral horn on postnatal day 2. At postnatal day 10-12, cords injected with HSVnr2d or HSVlac were removed and examined to compare the level of NR2D subunit mRNA expression, the degree of Mg^{2+} blockade of NMDAR and the action of NT-3 on monosynaptic transmission at sensory and descending synapses. As shown in FIG. 2, delivery of HSVnr2d enhanced the level of expression of NR2D subunit 1.2 fold ($p<0.001$) compared to controls injected with HSVlac. Up-regulation of NR2D

subunit expression was associated with the decrease of the degree of Mg^{2+} blockade (FIG. 2A). Interestingly, in 10-12 day old animals injected with HSVnr2d the degree of the Mg^{2+} block of NMDAR-mediated DR-responses (KMg = 1245 μM) and VLF-responses (KMg = 891 μM) decreased approximately to that detected at E18. In contrast, motoneurons recorded from the control cords injected with HSVlac did not exhibit Mg^{2+} blockade significantly different from that in untreated 2 w.o. animals (KMg = 741 μM for DR and KMg = 433 μM for VLF). Importantly, enhanced expression of NR2D in 2 w.o. animals restored the action of NT-3 on monosynaptic AMPA/kainate responses at both DR and VLF connections (FIGs. 2C and 2D).

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of treating a patient who has, or who has an elevated risk of developing, a sensory, motor, or cognitive deficit, the method comprising administering, to the patient, a vector comprising a sequence encoding a therapeutically effective amount of a subunit of an NMDA receptor (NMDAR) or a biologically active variant thereof.

2. The method of claim 1, wherein the patient is a mammal.

3. The method of claim 2, wherein the mammal is a human.

4. The method of claim 1, wherein the subunit is an NMDAR 2 (NMDAR2) subunit.

5. The method of claim 4, wherein the NMDAR2 subunit is NR2D.

6. The method of claim 1, wherein the vector is a nucleic acid vector.

7. The method of claim 6, wherein the nucleic acid vector is a plasmid.

8. The method of claim 1, wherein the vector is a viral vector.

9. The method of claim 8, wherein the viral vector is a retroviral vector.

10. The method of claim 1, wherein the vector is a herpes simplex virus amplicon.

11. The method of claim 1, wherein administering the vector comprises contacting a population of neuronal cells with the vector.

12. The method of claim 11, wherein the population comprises neuronal cells that naturally express an NMDAR.

13. The method of claim 11, wherein the population comprises neuronal cells that naturally express a receptor for neurotrophin-3 (NT-3).

14. The method of claim 11, wherein the population comprises motoneurons.

15. The method of claim 14, wherein the motoneurons are anterior horn motoneurons.

16. The method of claim 11, wherein the population comprises neurons whose axons or dendrites project through the spinal cord but whose cell somata lie elsewhere.

17. The method of claim 16, wherein the cell somata lie in the corticospinal tract or rubrospinal tract.

18. The method of claim 1, wherein the deficit is a motor deficit.

19. The method of claim 1, wherein the deficit is a sensory deficit.

20. The method of claim 1, wherein the deficit is a cognitive deficit.

21. The method of claim 20, wherein the cognitive deficit is manifest as an impaired memory or ability to learn.

22. The method of claim 20, wherein the cognitive deficit is manifest as dementia.

23. The method of claim 1, wherein the patient has, or has an elevated risk of developing, a traumatic injury to the spinal cord.

24. The method of claim 1, wherein the patient has, or has an elevated risk of developing, Alzheimer's disease, Parkinson's disease, or Huntington's disease.

25. The method of claim 1, wherein the patient has, or has an elevated risk of developing, Alpers' Disease, cerebral palsy, corticobasal ganglionic degeneration (CBD), progressive supranuclear palsy (PSP), Freidreich's ataxia, Lewy body dementia (LBD), multiple system atrophy (MSA), an idiopathic acquired ataxia, postpoliomyelitis syndrome, a prion disease, Rett syndrome, or tuberous sclerosis.

26. The method of claim 1, wherein the patient has, or has an elevated risk of developing, amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA).

27. The method of claim 1, wherein the patient has a birth defect involving the nervous system.

28. The method of claim 27, wherein the birth defect is hydrocephalus or spina bifida.

29. The method of claim 1, wherein the patient has, or has an elevated risk of developing, an age-related spinal myelopathy.

30. The method of any of claims 1-29, wherein the method further comprises administering a neurotrophin or a biologically active fragment thereof.

31. The method of claim 30, wherein the neurotrophin is administered by administering, to the patient, a vector comprising a sequence that encodes the neurotrophin or the biologically active fragment thereof.

32. The method of claim 30 or 31, wherein the neurotrophin is neurotrophin 3 (NT-3).

33. The method of any of claims 1-32, wherein administering the vector comprises applying the vector to a target tissue that has been surgically exposed.

34. A pharmaceutically acceptable composition comprising an expression vector comprising a sequence encoding a subunit of an NMDA receptor or a

biologically active variant thereof and, optionally, a physiologically acceptable carrier, diluent, or excipient.

35. The composition of claim 34, wherein the sequence is that of a subunit of a human NMDA receptor.

36. The composition of claim 34, wherein the sequence encodes a subunit of a human NMDA receptor that includes at least one amino acid substitution.

37. The composition of claim 34, wherein the sequence encodes a subunit of a human NMDA receptor that includes at least one amino acid deletion or addition.

38. The composition of claim 36 or claim 37, wherein no more than 10% of the amino acid residues within the subunit are substituted, deleted, or added.

39. The composition of any of claims 34-38, wherein the expression vector is an amplicon.

40. The composition of claim 39, wherein the amplicon is a herpes simplex virus amplicon.

41. The composition of claim 38, wherein the subunit is an NMDAR 2 (NMDAR2) subunit.

42. The composition of claim 40, wherein the NMDAR2 subunit is NR2D.

43. A pharmaceutically acceptable composition comprising an expression vector comprising a sequence encoding NT-3 or a biologically active variant thereof and, optionally, a physiologically acceptable carrier, diluent, or excipient.

44. The composition of claim 43, wherein the sequence is that of a human NT-3.

45. The composition of claim 43, wherein the sequence encodes a human NT-3 that includes at least one amino acid substitution.

46. The composition of claim 43, wherein the sequence encodes a human NT-3 that includes at least one amino acid deletion or addition.

47. The composition of claim 45 or claim 46, wherein no more than 10% of the amino acid residues within the NT-3 are substituted, deleted, or added.

48. The composition of any of claims 43-47, wherein the expression vector is an amplicon.

49. The composition of claim 48, wherein the amplicon is a herpes simplex virus amplicon.

50. The composition of any of claims 34-49, wherein the composition comprises an amplicon plasmid comprising an HSV origin of replication, an HSV cleavage/packaging signal, and a heterologous transgene expressible in a neuronal cell, wherein the transgene encodes a subunit of an NMDA receptor or a biologically active variant thereof or an NT-3 or a biologically active variant thereof.

51. The composition of claim 50, further comprising one or more vectors that, individually or collectively, encode all essential HSV genes but exclude all cleavage/packaging signals.

52. The composition of claim 51, further comprising a vector encoding an accessory protein.

53. A composition for use as a medicament in treating a patient who has, or who is at risk for, neural damage, wherein the composition comprises

(a) an amplicon plasmid comprising an HSV origin of replication, an HSV cleavage/packaging signal, and a heterologous transgene expressible in the host cell,

(b) one or more vectors that, individually or collectively, encode all essential HSV genes but exclude all cleavage/packaging signals, and

(c) a vector encoding an accessory protein, wherein the transgene encodes a subunit of an NMDA receptor or a biologically active variant thereof or an NT-3 or a biologically active variant thereof.

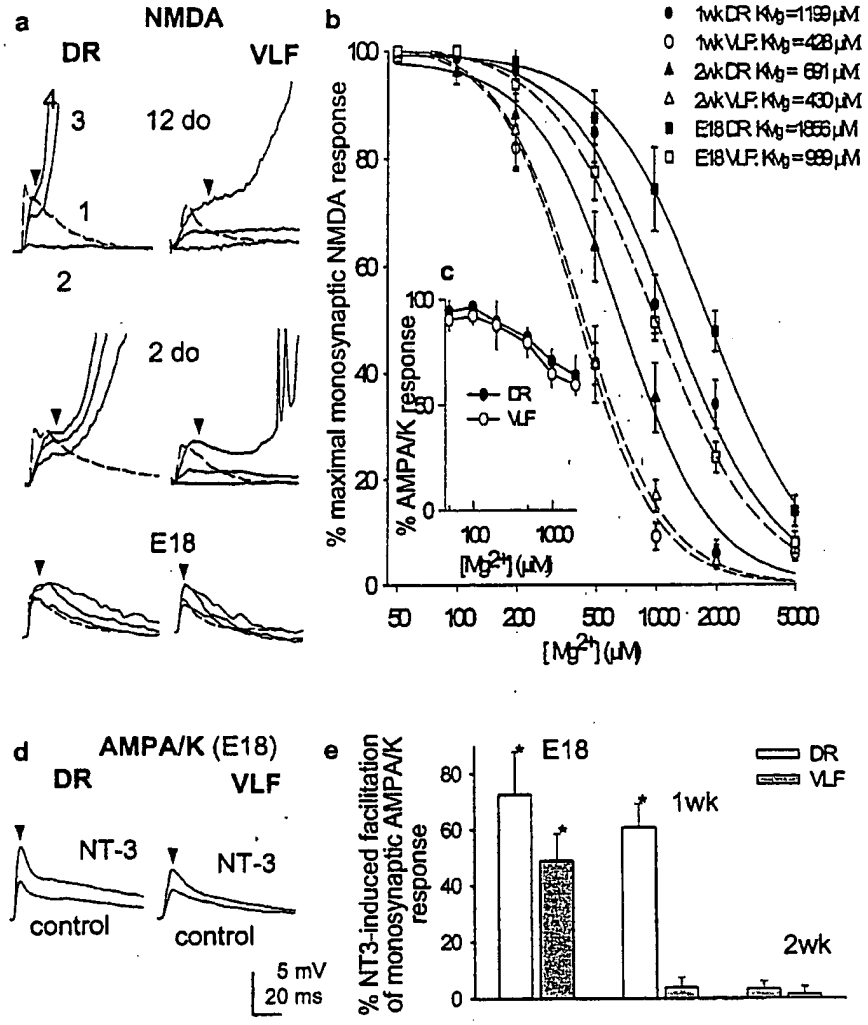
a
NMDA
DR
VLF
3
12 do
1
2
2 do
E18
b
1wk DR Kd=1199 μM
1wk VLF Kd=428 μM
2wk DR Kd= 691 μM
2wk VLF Kd=430 μM
E18 DR Kd=1855 μM
E18 VLF Kd=999 μM
% maximal monosynaptic NMDA response
[Mg2+] (μM)
c
% AMPA/K response
DR
VLF
[Mg2+] (μM)
d
AMPA/K (E18)
DR
VLF
NT-3
control
NT-3
control
5 mV
20 ms
e
% NT3-induced facilitation of monosynaptic AMPA/K response
E18
1wk
2wk
DR
VLF

FIG. 1

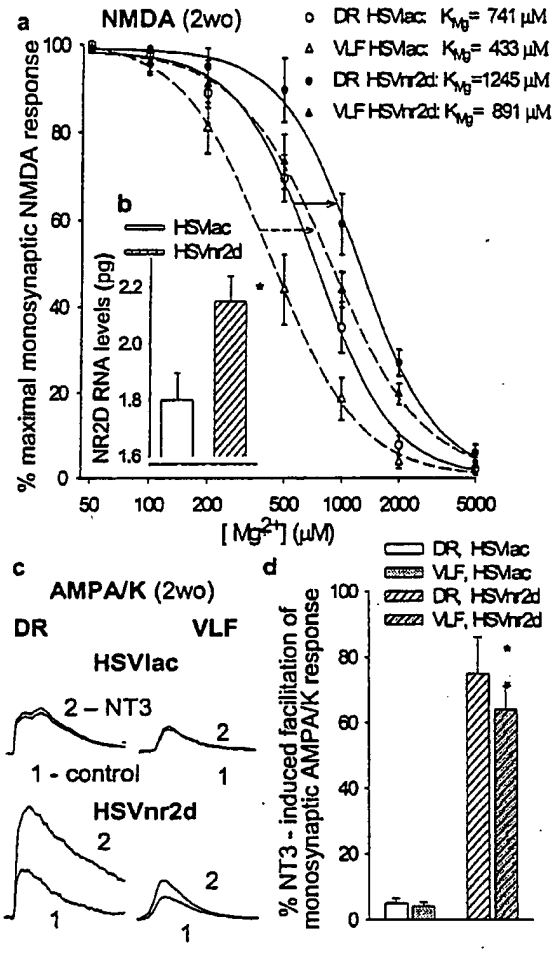
a
NMDA (2wo)
DR HSVlac: K_{Mg}= 741 µM
VLF HSVlac: K_{Mg}= 433 µM
DR HSVnr2d: K_{Mg}=1245 µM
VLF HSVnr2d: K_{Mg}= 891 µM
% maximal monosynaptic NMDA response
[Mg^{2+}] (µM)
b
HSVlac
HSVnr2d
NR2D RNA levels (pg)
c
AMPA/K (2wo)
DR
VLF
HSVlac
2 – NT3
1 - control
HSVnr2d
d
DR, HSVlac
VLF, HSVlac
DR, HSVnr2d
VLF, HSVnr2d
% NT3 - induced facilitation of monosynaptic AMPA/K response

FIG. 2

Table 1: Essential HSV-1 Genes

Gene*	Protein(Function)	Genbank I.D. No.	Genbank Accession No.**
UL1	virion glycoprotein L (gL)	136775	CAA32337
UL5	component of DNA helicase-primase complex	74000	CAA32341
UL6	minor capsid protein	136794	CAA32342
UL7	unknown	136798	CAA32343
UL8	DNA helicase/primase complex associated protein	136802	CAA32344
UL8.5	unknown***		
UL9	ori-binding protein	136806	CAA32345
UL15	DNA cleavage/packaging protein	139646	CAA32330
UL17	tegument protein	136835	CAA32329
UL18	capsid protein, VP23	139191	CAA32331
UL19	major capsid protein, VP5	137571	CAA32332
UL22	virion glycoprotein H, gH	138315	CAA32335
UL25	DNA packaging virion protein	136863	CAA32317
UL26	serine protease, self-cleaves to form VP21 & VP24	139233	CAA32318
UL26.5	capsid scaffolding protein, VP22a	1944539	CAA32319
UL27	virion glycoprotein B, gB	138194	CAA32320
UL28	DNA cleavage and packaging protein, ICP18.5	124088	CAA32321
UL29	single-stranded DNA binding protein, ICP8	118746	CAA32322
UL30	DNA polymerase	118878	CAA32323
UL31	UL34-associated nuclear protein	136875	CAA32324
UL32	cleavage and packaging protein	136879	CAA32307
UL33	capsid packaging protein	136883	CAA32308
UL34	membrane-associated virion protein	136888	CAA32309
UL36	very large tegument protein, ICP1/2	135576	CAA32311
UL37	tegument protein, ICP32	136894	CAA32312
UL38	capsid protein, VP19C	418280	CAA32313
UL42	DNA polymerase accessory protein	136905	CAA32305
UL48	alpha trans-inducing factor, VP16	114359	CAA32298
UL49	putative microtubule-associated protein, VP22	136927	~~CAA32299~~
UL49.5	membrane-associated virion protein	1944541	CAA32300
UL52	component of DNA helicase/primase complex	136939	CAA32288
UL54	regulation and transportation of RNA, ICP27	124180	CAA32290
α4 (*RS1*)	positive and negative gene regulator, ICP4	124141	CAA32286 CAA32278
US6	virion glycoprotein D, gD	73741	CAA32283

The complete genome of HSV-1 is reported at Genbank Accession No. X14112, which is hereby incorporated by reference in its entirety.

** Each of the listed Accession Nos. which report an amino acid sequence for the encoded proteins is hereby incorporated by reference in its entirety.

*** UL8.5 maps to a transcript which overlaps and is in frame with the carboxyl terminal of UL9 (Baradaran et al., "Transcriptional analysis of the region of the herpes simplex virus type 1 genome containing the UL8, UL9, and UL10 genes and identification of a novel delayed-early gene product, OBPC," J. Virol. 68(7):4251-4261 (1994), which is hereby incorporated by reference in its entirety).

FIG. 3

Gene bank No	Common Name	Gene Chip		RT-PCR	
		Fold change	"P" value	Fold change	"P" value
L08228	NR1	1.1	0.5	1.02	0.2
AF001423	NR2A	-1.3	0.7		
U11419	NR2B	-1.1	0.3		
U08259	NR2C	1.3	0.2		
U08260*	**NR2D**	**-1.8***	**0.05**	**-1.3***	**< 0.001**
U29873	NMDA-L	-1.4	0.03		

FIG. 4

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(54) Title: PREVENTION OR TREATMENT OF DEFICITS THAT ARISE IN CONNECTION WITH DISEASES OF, OR INJURIES TO, THE NERVOUS SYSTEM

(57) Abstract: The present invention includes compositions and methods for use in, for example, maintaining or increasing synaptic plasticity, strengthening synaptic transmission, and/or treating or preventing motor, sensory, or cognitive deficits (including enhancing memory and learning). The compositions and methods of the invention can be used to treat, for example, injuries to the nervous system (such as spinal cord injuries), a variety of neural and neurodegenerative diseases, including those associated with dementia (e.g., Alzheimer's disease), and disorders that interfere with learning or impair memory.

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INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01N 43/04, 63/00, 65/00; A61K 31/70
US CL : 514/44; 424/93.1
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 424/93.1

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, EMBASE, SCISEARCH, WPIDS, CAPLUS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARVIAN. V.L. et al. Removal of NMDA Receptor Mg+2 Block Extends the Action of NT-3 on Synaptic Transmission in Neonatal Rat Motoneurons. J. Neurophysiol. July 2001, Vol. 86. No. 1, pages 123-129, especially page 123.	1-6,8,10-18,23,26,30-23,45-49
X --- Y	HAASE G. et al. Gene Therapy of Murine Motor Neuron Disease using Adenoviral Vectors for Neurotrophic Factors. April 1997, Vol. 3. No. 4, pages 429-436, especially page 429.	43-44 --- 1-6,8,10-18,23,26,30-23,45-49
X --- Y	MARSH. D.R. et al. Herpes Simplex Viral and Amplicon Vector-Mediated Gene Transfer into Glia and Neurons in Organotypic Spinal Cord and Dorsal Root Ganglion Cultures. Molec. Therap. May 2000, Vol 1. No. 5, pages 464-478.	34-35, 39-40, 50-53 --- 1-6,8,10-18,23,26,30-32,41-42,45-49

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